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THE RELATIONSHIP BETWEEN CARCINOGENIC ACTIVITIES OF POLYCYCLIC AROMATIC HYDROCARBONS AND THEIR SINGLET, TRIPLET, AND SINGLET–TRIPLET SPLITTING ENERGIES AND PHOSPHORESCENCE LIFETIMES

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Abstract—The energies of the lowest excited singlet, E_s, and triplet, E_t, states, and singlet–triplet splitting energies, $\Delta E_{s,t}$, were determined on 18 carcinogenic and 31 noncarcinogenic polycyclic aromatics. A highly significant correlation was found between carcinogenic activity and the energy of the excited singlet state. Compounds with an $E_s < 312$ kJ/mol were 4.8 times more likely to be carcinogens than those compounds with $E_s \geq 312$ kJ/mol ($P = 0.015$). Compounds whose singlet energies fell within the narrow range of $297 \leq E_s \leq 310$ kJ/mol were 22.8 times more likely to be carcinogens than those compounds which fell outside this range ($P = 0.00006$). A significant correlation between carcinogenic activity and E_t energies was not found, while the correlation involving $\Delta E_{s,t}$ energies was intermediate between the E_s and E_t correlations. The phosphorescence lifetimes, τ_p, of the 18 carcinogenic aromatics and 27 of the noncarcinogenic aromatics were determined, and were shown not to be correlated with carcinogenic activity. When either the E_t or $\Delta E_{s,t}$ energies were plotted as a function of E_s it was found that the carcinogens tended to form in an elliptical cluster. Compounds whose E_s and E_t energies placed them within the ellipse were 9.7 times more likely to be carcinogens than those compounds which fell outside the ellipse ($P = 0.002$), while with the E_s, $\Delta E_{s,t}$ ellipse, compounds which fell inside were 20.6 times more likely to be carcinogens than those which fell outside ($P = 0.0004$). E_s, E_t, $\Delta E_{s,t}$, and τ_p values were also determined on 12 carcinogenic and 4 noncarcinogenic alkyl substituted benz[a]anthracenes. There was no significant difference between the carcinogens and noncarcinogens and the "elliptical" correlation predicted both the carcinogens and noncarcinogens to be carcinogenic. The results suggest that either some property(ies) of the lowest excited singlet state, but not its energy, or some molecular property(ies) which runs parallel to singlet state energies may be important in determining carcinogenic activity in polycyclic aromatics.

INTRODUCTION

The carcinogenic polycyclic aromatic hydrocarbons and their carcinogenic heterocyclic analogs (herein jointly referred to as PAH‡) include a large number of compounds which are essentially planar and usually contain four or more fused rings. One of the more active areas of research on these chemical carcinogens has been the attempt to find some correlation between carcinogenic activities and some property common to these compounds. Most researchers generally agree that one of the early events in the carcinogenic process is a reaction between certain cellular substrates (i.e. nucleic acids and/or proteins) and the carcinogen. The possibility that such a reaction could involve electronic excited states was first considered by Heiger (1930). Work by Jones (1940) showed a rough correlation between carcinogenic activity and the energy of Clar's β-band (Clar, 1964a) in benz[a]anthracene and some of its derivatives, while the work of Moodie and Reed (1954) showed that there was no correlation between the energy of the lowest excited triplet state of some closely related PAH and their carcinogenic activities. Birks (1961), however, suggested that a good correlation existed between the energy of the first excited singlet state of PAH and their carcinogenicities and proposed that the first step in chemical carcinogenesis might involve the binding of the PAH to protein followed by resonance energy transfer from the protein to the PAH. Mason (1958a, 1958b) considered carcinogenesis as an electron transfer process from a protein to an unoccupied orbital in the carcinogen and found a limited correlation between molecular electronic transition energies calculated by the Hückel method and carcinogenic activity. Sung and Lazar (1966) found that there was no significant difference in the average energies of Clar's α-, p-, and β-bands and

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‡ Abbreviations used: BAP, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; PAH, polycyclic aromatic hydrocarbons.

observed that variation of these energies within the carcinogens was significantly less than within the noncarcinogens.

The formation of excited states during the metabolism of PAH was first suggested by Anderson (1947). He theorized that the exothermicity of the metabolic hydroxylation of the carcinogen was sufficient to excite the carcinogen to a higher electronic state. The fluorescence emitted by the metabolically generated excited state could then be absorbed by labile cellular components resulting in a photochemical transformation which subsequently led to malignancy. Steele (1966) extended Anderson's theory and postulated that an excited state of the carcinogen, produced metabolically, could produce hydroxyl radicals from hydrogen peroxide and that these radicals then entered into the cancer producing steps. Buu-Hoï and Sung (1970) and Sung and Buu-Hoï (1970) proposed a nonradiative photochemical model for PAH carcinogenesis in which light was not necessary. An excited state of the carcinogen was proposed to be produced metabolically and then undergo photochemical reaction with cellular substrates to initiate the carcinogenic process. To substantiate this hypothesis Buu-Hoï and Ba Giao (1971) treated mice with perdeuterated 7,12-dimethylbenz[*a*]anthracene (DMBA) and non-deuterated DMBA. Substitution of deuterium for hydrogen would lengthen the lifetime of the triplet excited state of the carcinogen and thereby increase its likelihood to undergo photochemical reactions with cellular components. The results showed nearly a two-fold increase in carcinogenic activity of the perdeuterated compound.

The initiation of PAH carcinogenesis by light has also been investigated. Ts'o and Lu (1964) reported that the chemical linkage of benzo[*a*]pyrene (BAP) to DNA could be induced photochemically. More recent work by Hoffman and Müller (1969), Cavalieri and Calvin (1971) and Blackburn *et al.* (1972) have also shown that BAP reacts photochemically with nucleic acid bases. Covalent binding occurs at the six position in BAP, and Moore *et al.* (1973) have shown, from calculated reactivity indexes, that the six position is the most reactive for the triplet excited state of BAP. Blackburn *et al.* (1973) found that carcinogenic and noncarcinogenic PAH photoreacted differently with DNA. *In vivo* experiments in mice by Santamaria *et al.* (1966) have shown that BAP was carcinogenic independent of light, but that its carcinogenicity could be influenced by light with either acceleration or inhibitiion with respect to dark controls. Cavalieri and Calvin (1971) have also shown that light could enhance the carcinogenic activity of BAP on mice, and that the noncarcinogens, benzo[*e*]pyrene, chrysene, and pyrene were still noncarcinogenic in animals exposed to light.

Further evidence that electronic excited states may be involved in PAH carcinogenesis has come from the work of Seliger *et al.* (1974) and Stauff *et al.* (1973). These workers have shown the presence of electronic excited states in cigarette smoke and have suggested that these may be excited states of PAH. Finally, the significant correlations between photodynamic activity and carcinogenicity (Epstein *et al.*, 1964a; Morgan and Warshawsky, see the following paper) lends further support to the theory that excited states are involved in PAH carcinogenesis.

In this work we have determined the energies of the lowest excited singlet and triplet states, and singlet–triplet splitting energies of 18 carcinogenic and 31 noncarcinogenic PAH. Phosphorescence lifetimes have also been determined on the carcinogens and 27 of the noncarcinogens. The statistical association of these photophysical properties with carcinogenic activity was determined and we find a highly significant association between carcinogenic activity and the energy of the lowest excited singlet state of PAH.

MATERIALS AND METHODS

*Compounds**. Acridine, anthracene, benz[*c*]acridine, benz[*a*]anthracene, benzo[*ghi*]fluoranthene, 11H-benzo[*a*]fluorene, 10H-benzo[*b*]fluorene, benzo[*rst*]pentaphene, benzo[*a*]pyrene, benzo[*e*]pyrene, benzo[*b*]triphenylene, carbazole, coronene, dibenz[*a,e*]aceanthrylene†, dibenzo[*def,mno*]chrysene, diindeno[1,2,3-*cd*:3,2,1-*lm*]perylene, fluoranthene, fluorene, naphthacene, ovalene, 7-oxobenz[*de*]anthracene, picene, pyrene, and triphenylene were obtained from Aldrich Chemical Co.: dibenz[*a,h*]anthracene, and 7H-dibenzo[*c,g*]carbazole were obtained from K & K Laboratories: dibenz[*a,h*]acridine and dibenz[*a,j*]acridine were obtained from Sigma Chemical Co.: and phenazine and perylene were supplied by Pflantz and Bauer.

Benz[*a*]acridine (Tada *et al.*, 1961), benz[*e*]acephenanthrylene (Thiele and Henle, 1906; and Sieglitz, 1919), 11H-benzo[*a*]carbazole (Ghighi, 1930; and Borsch *et al.*, 1907), benzo[*k*]fluoranthene (Buu-Hoi *et al.*, 1959; Johnson, 1963; and Brande *et al.*, 1954), dibenz[*a,c*]acridine (Adelfang and Cromwell, 1961), dibenzo[*g,p*]chrysene (Clar *et al.*, 1964b), dibenzo[*h,rst*]pentaphene (Zinke *et al.*, 1952), and naphtho[1,2,3,4-*def*]chrysene (Schiedt, 1938) were synthesized using known literature procedures. Chrysene, benzo[*c*]chrysene, benzo[*g*]chrysene, benzo[*c*]phenanthrene, benzo[*ghi*]perylene, dibenz[*a,j*]anthracene, and phenanthrene were prepared by the photocyclization of the appropriate diarylethylene under oxidative conditions (Wood *et al.*, 1964; Dietz and Scholz, 1968; Laarhoven *et al.*, 1970; Morgan *et al.*, 1970). Benzo[*h*]chrysene and dibenzo[*a,c*]phenazine were obtained from Dr. S. H. Horgan. 7H-benzo[*c*]fluorene was a gift from Professor M. Orchin, and phenanthro[3,4-*c*]phenanthrene and the methyl substituted benz[*a*]anthracenes were provided by Professor M. S. Newman. All of the above compounds were purified by either recrystallization, chromatography, or sublimation or by a combination of these methods.

Preparation of samples. Fluorescence emission spectra were obtained on solutions approximately 10 μM in PAH in either spectrograde methanol or 95% ethanol. Samples

* The names used throughout this work comply with the nomenclature used by Chemical Abstracts-Index of Ring Systems.

† This compound was purchased as "dibenzo[*a,l*]pyrene", but was found to actually be dibenz[*a,c*]aceanthrylene (see: Lacassange *et al.*, 1968).

for phosphorescence emission spectra and phosphorescence lifetimes were prepared by dissolving the PAH in EA (2 parts anhydrous ether, 1 part 95% ethanol) in quantities such that the final concentrations were between 0.1 and 1.0 m*M*. A small volume (0.4 m*l*) of these solutions were placed in Pyrex tubes, 5 mm OD, which had a standard taper joint attached to them. The samples were degassed on a high vacuum line using five freeze-pump-thaw cycles at 10^{-6} torr, and then permanently sealed.

Emission spectra and lifetimes. The fluorescence emission spectrum of each compound was obtained on an Aminco Bowman Spectrophotofluorometer which had the excitation monochromator removed. A Coherent Radiation Laboratories Model 53 Argon Laser with emission wavelengths at 350 and 365 nm was used as the exciting source. Each spectrum was recorded on an x–y recorder. The phosphorescence spectrum of each compound was obtained by mounting an Aminco phosphoroscope in the above instrument. The laser radiation was passed through the lower part of a quartz Dewar flask which contained the sample tube at 77 K. The emitted light was detected by either a RCA 1P28 or 7102 photomultiplier tube.

The phosphorescence lifetimes were obtained by making the following modifications on the Aminco instrument. The rotating shutter was removed from the phosphoroscope and an Avco Everett Research Laboratory Pulsed Nitrogen Laser, Model C950, replaced the argon laser. The signal coming from the photomultiplier tube, following a laser pulse, was passed through the electrometer circuit of the Aminco Microphotometer and then through a voltage amplifier which had a range of 0 to −10 V. The decay signal was displayed on a Tektronix 7704 oscilloscope and simultaneously sent over hardwired, dedicated phone lines to a Raytheon 704 minicomputer by the voltage amplifier. All of the data acquisition and reduction was handled by the computer, which had the capacity of acquiring 1400 points per buffer and could acquire the data at a rate of 8–32,000 μs between points. Data acquisition was initiated from the emission signal using a preset voltage of −5.0 V (i.e. when the emission signal reached −5.0 V the computer began acquiring data). Decay signals were taken 4–64 times (depending on the signal to noise ratio) for each compound, and then averaged to give the decay curves from which the phosphorescence lifetimes were determined.

The data in the early portion of each decay curve was eliminated since the initial portion of these curves contained both scattered laser light and fluorescence from the compounds. Data which were acquired after the phosphorescence decay had decreased to 5% of its initial value also were not used in the calculation of lifetimes. Once the exponential phosphorescence decay curve for each compound had been determined the computer determined the phosphorescence lifetimes by the method of least squares. The proper time constants for our circuit were determined, for each compound, by changing the resistor and/or capacitor settings on the voltage amplifier and then redetermining the phosphorescence lifetime. This process was repeated until there was no further decrease in the calculated phosphorescence lifetime. Linear correlation coefficients of at least 0.999 were obtained for the decay curves of each compound.

RESULTS

The energies of the lowest excited singlet, E_s, and triplet, E_t, states were determined for 49 different polycyclic aromatic ring systems from the 0-0 band in their fluorescence and phosphorescence emission spectra, respectively. The singlet-triplet splitting energies, $\Delta E_{s,t}$, were obtained from the difference between E_s and E_t for each compound. Phosphorescence lifetimes, τ_p, were determined on 45 of the above compounds using a computer assisted technique similar to that described by Mathiasch (1971). The E_s, E_t, $\Delta E_{s,t}$, and τ_p values were also determined for a family of compounds, the methyl substituted benz[*a*]anthracenes. The division of the compounds into carcinogens and noncarcinogens was made on the basis of the animal test data of Hartwell (1951), Shubik and Hartwell (1957 and 1969), and Tracor Jitco (1973a, 1973b, and 1973c). The energies, lifetimes, and carcinogenic activities of the 49 ring systems and the methyl substituted benz[*a*]anthracenes are listed in Tables 1 and 2, respectively.

The statistical method developed by Cornfield (1951), the so-called "relative odds" method (Epstein *et al.*, 1964a), was used to determine the correlation between E_s, E_t, $\Delta E_{s,t}$, and τ_p values and carcinogenic activity. Two approaches were used in analyzing the data. In the first a single value of energy or lifetime was considered, above or below which a compound would possess carcinogenic activity (single value cut-off). In the second a range of energy or lifetime values was found in which carcinogenic activity was most prevalent (range of values). The values chosen in both of these methods were those which gave the best statistical correlation. The reliability of the results obtained from the "relative odds" method were then tested using Fisher's exact test (Langley, 1971; Brownlee, 1965). The results of these correlations showed that E_s values are strongly correlated with carcinogenicity (Table 3), but E_t, $\Delta E_{s,t}$, and τ_p values are not.

When either E_t or $\Delta E_{s,t}$ values were plotted as a function of E_s it was found that the carcinogenic compounds tended to group in a cluster whose boundaries were most conveniently represented by an ellipse. The E_s, E_t ellipse (Fig. 1a) has foci (E_s, E_t) at 280.3, 173.6 and 322.2, 232.2 kJ/mol, a principal axis equal to 86.2 kJ/mol, and is represented by Eq. 1.

$$[(E_s - 280.3)^2 + (E_t - 173.6)^2]^{1/2} + [(E_s' - 322.2)^2 + (E_t - 232.2)^2]^{1/2} \leq 86.2 \quad (1)$$

Thus, a compound would fall inside or on the boundary of the ellipse if the left hand side of Eq. 1 is less than or equal to 86.2. Similarly, the E_s, $\Delta E_{s,t}$ ellipse (Fig. 1b) has foci (E_s, $\Delta E_{s,t}$) at 285.8, 112.1 and 331.4, 82.4 kJ/mol, a principal axis equal to 72.8 kJ/mol, and is represented by Eq. 2.

$$[(E_s - 285.8)^2 + (\Delta E_{s,t} - 112.1)^2]^{1/2} + [(E_s - 331.4)^2 + (\Delta E_{s,t} - 82.4)^2]^{1/2} \leq 72.8 \quad (2)$$

The ellipse in each case was the one which gave the best correlation with carcinogenic activity. The statistical analyses for these ellipses are given in Table 4. No statistically significant correlations with carcino-

Table 1. Singlet, triplet and singlet–triplet splitting energies, and phosphorescence lifetimes[a]

Compound	Number of rings†	E_s	E_t	E_t (lit)[b]	$\Delta E_{s,t}$	τ_p	τ_p (lit)[b]
Carcinogens							
1. Benz[*a*]anthracene	4	310	200	197[e]	110	0.359	0.3[e] 0.4[d]
2. 7-Oxobenz[*de*]anthracene	4[O]	303	192		111	0.024	
3. Benzo[*a*]pyrene	5	297	177	176[e] 178[f]	120	0.105	0.11[f]
4. 11H-Benzo[*a*]carbazole	4[N]	341	256	252[g] 248[h]	85	4.74	3.6[g]
5. Benz[*e*]acephenanthrylene	5	328	229	230[i]	99	1.80	2.0[i]
6. Benzo[*c*]chrysene	5	310	233		77	1.53	
7. Benzo[*c*]phenanthrene	4	321	239	240[j] 238[k]	82	3.34	3.5[j]
8. Benzo[*g*]chrysene	5	322	226		96	1.46	
9. Benzo[*k*]fluoranthene	5	299	211		88	0.828	
10. Benzo[*rst*]pentaphene	6	276	169	169[l]	107	0.052	
11. Dibenz[*a,h*]acridine	5[N]	306	229		77	2.31	
12. Dibenz[*a,h*]anthracene	5	303	218	219[j]	85	1.60	1.5[e,m]
13. Dibenz[*a,j*]acridine	5[N]	303	223	222[j]	80	1.04	
14. Dibenz[*a,j*]anthracene	5	303	221		82	2.51	
15. Dibenz[*a,e*]aceanthrylene	6	252	196		56	0.558	
16. Naphthio[1,2,3,4-*def*]chrysene	6	302	197	196[n]	105	0.574	
17. 7H-Dibenzo[*c,g*]carbazole	5	304	231	235[g]	73	2.25	2.1[g]
18. Dibenzo[*h,rst*]pentaphene	7	286	193		93	0.499	
Noncarcinogens							
19. Acridine	3[N]	315	190		125	0.155	
20. Dibenzo[*def,mno*]chrysene	6	276	143		133		
21. Anthracene	3	319	178	176[l]	141		0.09[o]
22. Benz[*a*]acridine	4[N]	312	213		99	0.406	
23. Benz[*c*]acridine	4[N]	312	213		99	0.281	
24. 11H-Benzo[*a*]fluorene	4	326	241		85	2.61	
25. 10H-Benzo[*b*]fluorene	4	326	240		86	2.24	
26. 7H-Benzo[*c*]fluorene	4	332	231		101	1.14	1.1[l]
27. Benzo[*b*]chrysene	5	305	190		115	0.185	
28. Benzo[*b*]triphenylene	5	320	213	212[j]	107	0.794	
29. Benzo[*e*]pyrene	5	327	221	224[f] 222[j]	106	2.12	1.86[f] 2.0[p]
30. Benzo[*ghi*]fluoranthene	5	282	226	227[l]	56	0.378	
31. Benzo[*ghi*]perylene	6	294	193	194[f] 194[j]	101	0.438	0.44[f]
32. Carbazole	3[N]	355	293	294[q]	62	8.04	14.5[m] 10.0[q]
33. Chrysene	4	332	239	240[j]	93	2.54	2.5[e]
34. Coronene	7	279	232	229[e]	47	9.5	9.4[e]
35. Dibenz[*a,c*]acridine	5[N]	322	227		95	0.741	
36. Dibenzo[*a,c*]phenazine	5[NN]	296	223		73	0.286	
37. Dibenzo[*g,p*]chrysene	6	312	207		105	0.821	
38. Fluoranthene	4	295	221	222[j]	74	0.990	0.85[r] 0.9[p]
39. Fluorene	3	397	284		113		5.0[f,s] 4.3[m]
40. Phenanthro[3,4-*c*]phenanthrene	6	290	228	228[t]	62	1.86	2.1[t]
41. Naphthacene	4	254		123[o]	131		0.005[o]
42. Ovalene	10	256	184		72		
43. Diindeno[1,2,3-*cd*,3,2,1-*lm*]-perylene	9	221	146		75		
44. Perylene	5	275		148[u]	127		
45. Phenanthrene	3	346	259	259[v] 259[j]	87	2.94	3.5[m] 3.7[v]
46. Phenazine	3[N]	299	186	183[w]	113	0.083	
47. Picene	5	318	240	241[x]	78	2.7	2.5[x]
48. Pyrene	4	322	203	201[e] 202[x]	119	0.63	0.5[f,s] 0.7[x]
49. Triphenylene	4	352	282	285[y] 279[x]	70	15.2	15.9[x,m] 16.0[y]

Footnote on page 35.

Table 2. Singlet, triplet and singlet–triplet splitting energies, and phosphorescence lifetimes for benz[*a*]anthracene and some methyl substituted benz[*a*]anthracenes[a]

Compound	E_s	E_t	E_t (lit)	ΔE_{st}	τ_p	τ_p (lit)
Carcinogens						
Benz[*a*]anthracene	310	200	201[b] 197[c,d] 200[e]	110	0.359	0.3[d] 0.24[e]
4-Methyl-	310[f]	199	199[b] 200[c] 200[e]	111	0.298	0.27[e]
5-Methyl-	308[f]	202	202[b] 201[e]	106	0.383	
6-Methyl-	310[f]	203	201[b] 203[c] 200[e]	107	0.407	0.26[e]
7-Methyl-	308[f]	195	195[b] 195[c] 192[e]	113	0.219	0.15[e]
8-Methyl-	311[f]	201	201[b] 200[c] 197[e]	110	0.341	0.23[e]
9-Methyl-	311[f]	199	202[b] 198[e]	112	0.298	
10-Methyl-	309[f]	199	203[b] 199[c]	110	0.304	
12-Methyl-	306[f]	192	194[b] 195[c] 191[e]	114	0.142	0.078[e]
7,12-Dimethyl-	297	184	186[b]	113	0.110	
1,12-Dimethyl-	295	195		100	0.110	
6,8-Dimethyl-	308	203		105	0.385	
3-Methylcholanthrene	302	195		107	0.313	
Noncarcinogens						
1-Methyl-	310[f]	203	203[b] 203[c]	107	0.322	
2-Methyl-	309[f]	200	200[b] 199[c] 198[e]	109	0.323	0.23[e]
3-Methyl-	312[f]	201	200[b] 200[c] 199[e]	111	0.321	0.25[e]
11-Methyl-	310[f]	202	201[b] 201[c] 197[e]	108	0.358	0.24[e]

[a] Energy values are in kJ/mol, and lifetimes are in s. [b] Hirschberg, Y. (1956) *Anal. Chem.* 28, 1954–1957. [c] Moodie, M. M., and C. Reid (1954) *J. Chem. Phys.* 22, 252–254. [d] McClure, D. S. (1949) *J. Chem. Phys.* 17, 905–913. [e] Benson, R., and N. E. Geacintov (1973) *J. Chem. Phys.* 59, 4428–4434. (Lifetime values were obtained at 298 K in a polyacrylate matrix.) [f] Becker, R. S., I. S. Singh and E. A. Jackson (1964) *J. Chem. Phys.* 32, 2144–2170.

Footnote to Table 1

† Superscript indicates the nature of the heterocyclic compounds—e.g. 4^O is a tetracyclic compound with one oxygen atom. [a] Energy values are in kJ/mol and lifetimes are in seconds. [b] Representative literature values for the triplet state energies and phosphorescence lifetimes are included for the purpose of comparison and do not represent a complete review of the literature. Literature values for the singlet state energies are not included since these values are generally well known and accepted. [c] McClure, D. S. (1949) *J. phys. Chem.* 17, 905–913. [d] Czekalla, J., G. Griegleb, W. Herre and H. J. Vahlensieck (1959) *Z. Elecktro. Chem.* 63, 715–721. [e] Muel, B., and M. Hubert-Habart (1958) *J. Chim. Phys.* 55, 377–383. [f] Mathiasch, B. (1971) *Anal. Letters* 4, 519–529. [g] Zander, M. (1968) *Phosphorimetry*, p. 100. Academic Press, N.Y. [h] Zander, M. (1964) *Chem. Ber.* 97, 2695–2699. [i] Reference (g), p. 68. [j] Clar, E., and M. Zander (1956) *Chem. Ber.* 89, 749–762. [k] Moodie, M. M., and C. Reid (1954) *J. Chem. Phys.* 22, 252–254. [l] Giiiveman, O. L. J., F. Kaufman and G. Porter (1973) *J. Chem. Soc., Faraday Transactions II* 69, 708–737. [m] Dikum, P. P., A. A. Petrov and B. Y. Sveshnikov (1951) *Z. Eksp. Teoret. Fiz.* 21, 150–163. [n] Il'ina, A. A., and E. V Shpol'skii (1951) *Izv. Akad. Nauk S.S.S.R. Ser. Fiz.* 15, 585–595. [o] McGlynn, S. P., M. R. Padhyde and M Kasha (1954) *J. Chem. Phys.* 22, 593–594. [p] Reference (g), p. 36. [q] Heckman, R. C. (1958) *J. Mol. Spectrosc.* 2, 27–41. [r] Von Foerster, G. (1963) *Z. Naturforsch.* 18A, 620–626. [s] Zander, M. (1965) *Angew. Chem. Intern. Ed. Engl.* 4, 930–938. [t] Rhodes, W., and M. F. A. Elsayed (1962) *J. Mol. Spectrosc.* 9, 42–49. [u] Clarke, R. N., and R. M. Hochstrasser (1969) *J. Mol. Spectrosc.* 32, 309–319. [v] Lim, E. C., and J. D. Laposa (1964) *J. Chem. Phys.* 41, 3257–3259. [w] Lewis, G. N., and M. Kasha (1944) *J. Am. Chem. Soc.* 66, 2100–2116. [x] Langelaar, J., R. P. H. Rettschnick, and G. J. Hoytink (1971) *J. Chem. Phys.* 54, 1–7. [y] Craig, D. P., and I. G. Ross (1954) *J. Chem. Soc.* 1589–1606. [z] Azumi, T., and S. P. McGlynn (1963) *J. Chem. Phys.* 39, 1186–1194.

Table 3. Statistical analyses of the correlation between singlet state energy and carcinogenicity

a. Single value cut-off

	Singlet state energy (kJ/mol) $E_s < 312$	$E_s \geq 312$
Carcinogens	14	4
Noncarcinogens	13	18

The "relative odds" of a compound being carcinogenic if its $E_s < 312$ are $(14 \times 18)/(4 \times 13) = 4.8$, $P = 0.015$

b. Range of values

	Range of singlet state energy (kJ/mol) $297 \leq E_s \leq 310$	$310 < E_s < 297$
Carcinogens	11	7
Noncarcinogens	2	29

The "relative odds" of a compound being carcinogenic if $297 \leq E_s \leq 310$ are 22.8, $P = 0.00006$

genic activity were found when plots of E_s vs τ_p, E_t vs $\Delta E_{s,t}$, E_t vs τ_p and $\Delta E_{s,t}$ vs τ_p were considered.

DISCUSSION

The precise mechanism by which carcinogenic PAH transform normal cells into cancer cells is not known. The possibility that carcinogenesis could involve charge transfer complexes has been considered by Mulliken (1952), Badger (1954), and Dewar and Lepley (1961). However, Epstein *et al.* (1964b) and Van Duuren (1969) have presented strong evidence against a significant correlation between charge transfer complex formation and carcinogenicity. Most of the current theories of PAH carcinogenesis are based on the assumption that covalent binding of the PAH, or more likely one or more of its metabolites, to cellular material is necessary, and that such a process involves only ground state chemistry. The ground state reactivity indexes calculated by Pullman and Pullman (1955), Sung (1972), and Herndon (1974) have directed attention to reactions involving the molecular *K*- and *L*-regions in PAH. However, Moriconi and Salce (1968) have shown that a number of carcinogenic and noncarcinogenic PAH react with ozone in both molecular regions, but with product distributions that are not consistent with the calculated *K*- and *L*-region reactivities.

The study of the metabolism of PAH has been limited to a relatively small group of compounds. Products such as epoxides, dihydrodiols, phenols, and quinones have been identified. The *K*-region epoxides of these compounds have been suggested as being the actual carcinogen and in the case of the widely studied benzo[*a*]pyrene (BAP) it has been shown that the *K*-region epoxide is indeed formed by rat liver microsomes (Grover *et al.*, 1972), rat lung microsomes (Grover, 1974), and hamster liver microsomes (Wang *et al.*, 1972). However, recent work has shown that the binding of BAP to DNA produces products that

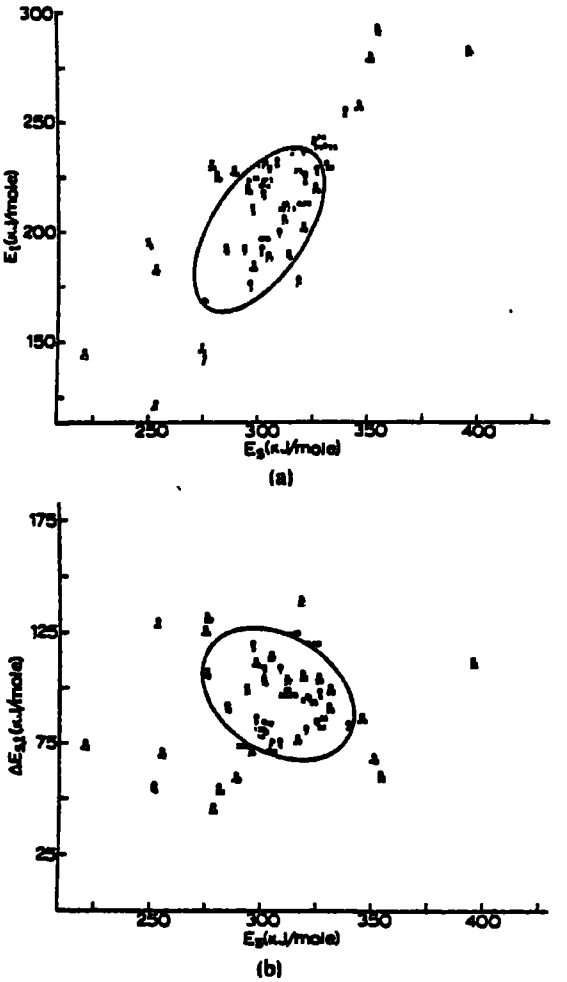

Figure 1. (a) Elliptical clustering of carcinogens as a function of their E_s and E_t energies. (b) Elliptical clustering of carcinogens as a function of their E_s and $\Delta E_{s,t}$ energies. Closed circles represent carcinogenic compounds.

are not identical to those formed when DNA is reacted with the *K*-region epoxide of BAP (King *et al.*, 1975; Baird *et al.*, 1975; Kodama and Nagata, 1975). Evidence has also been presented that indicates that the 1,3, and 6-positions are not involved in the enzyme catalyzed covalent binding of BAP to polyguanylic acid (polyG) (Meehan *et al.*, 1976a) and that the 6-position is not involved in the binding to DNA (King *et al.*, 1976a). Current evidence indicates that the metabolite of BAP that binds to DNA and polyG is a 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo-[*a*]pyrene (Osborne *et al.*, 1976; King *et al.*, 1976b; Meehan *et al.*, 1976b).

Regardless of whether PAH themselves or products of single or double metabolism are the ultimate carcinogens, the fact remains that there must be some critical activation step which carcinogenic PAH undergo. That such an activation could involve excited state chemistry has been proposed by Anderson (1947), Steele (1966), Buu-Hoï and Sung (1970), and Sung and Buu-Hoï (1970), and the recent work by Hamman and Seliger (1976) has demonstrated that excited states are indeed formed during the micro-

Table 4. Statistical analyses of the elliptical clustering of carcinogens with respect to excited state energies

a. E_s *vs* E_t *ellipse*

	Position in elliptical correlation	
	Inside ellipse	Outside ellipse
Carcinogens	16	2
Noncarcinogens	14	17

The "relative odds" of a compound being carcinogenic if it falls inside the E_s, E_t ellipse are 9.71. $P = 0.0024$

b. E_s *vs* $\Delta E_{s,t}$ *ellipse*

	Position in elliptical correlation	
	Inside ellipse	Outside ellipse
Carcinogens	17	1
Noncarcinogens	14	17

The "relative odds" of a compound being carcinogenic if it falls inside the E_s, $\Delta E_{s,t}$ ellipse are 20.6. $P = 0.0004$

somal hydroxylation of BAP. The photochemically induced binding of carcinogenic PAH to DNA and nucleic acids has been demonstrated by Ts'o and Lu (1964), Hoffman and Müller (1969), Cavalieri and Calvin (1971), and Blackburn *et al.* (1972, 1973), and acceleration of carcinogenesis by light has been found in BAP by Santamaria *et al.* (1966) and Cavalieri and Calvin (1971). These results suggest that electronic excited states may intervene at some critical point in PAH carcinogenesis.

The present work supports and extends the finding by Birks (1961) that a highly significant correlation exists between carcinogenic activity and the energy of the first excited singlet state of PAH (Table 3).

Compounds with an $E_s < 312$ kJ/mol were 4.8 times more likely to be carcinogens than those compounds with $E_s \geqslant 312$ kJ/mol, ($P = 0.015$). When a narrow range of singlet energies was considered it was found that a compound was 22.8 times more likely to be a carcinogen if $297 \leqslant E_s \leqslant 310$ kJ/mol. ($P = 0.00006$). A significant correlation between carcinogenic activity and either E_t, $\Delta E_{s,t}$, or τ_p values was not found except in the case where a range of $\Delta E_{s,t}$ values were considered. A compound was 6.9 times more likely to be a carcinogen if its $\Delta E_{s,t}$ energy fell in the range $76.6 \leqslant \Delta E_{s,t} \leqslant 110$ kJ/mol. ($P = 0.005$).

The "elliptical" correlations (Fig. 1) are highly significant (Table 4). Most of the carcinogenic "exceptions" in the correlations involving E_s were removed, but in so doing more of the noncarcinogens were placed in the "active" category. In the 16 alkyl substituted benz[*a*]anthracenes the "elliptical" correlation predicted both the carcinogens and noncarcinogens to be carcinogenic. These results suggest that either some property(ies) of the lowest excited singlet state, but not its energy, or some molecular property(ies) which runs parallel to singlet state energies may be important in determining carcinogenic activity in PAH. The lack of a correlation between carcinogenic activity and either E_t or τ_p values makes it seem unlikely that the lowest excited triplet state, or a molecular property associated with it, is involved in PAH carcinogenesis.

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THE METABOLISM OF 7*H*-DIBENZO[*c*,*g*]CARBAZOLE, AN *N*-HETEROCYCLIC AROMATIC, IN THE ISOLATED PERFUSED LUNG

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SUMMARY

The metabolism of a carcinogenic *N*-heterocyclic aromatic, 7*H*-dibenzo[*c*,*g*]carbazole, was investigated in an isolated perfused rabbit lung preparation and in rat liver microsomes. A major metabolite produced in both preparations is the 7-hydroxydibenzo[*c*,*g*]carbazole. A substantial percentage of this metabolite is found in the tracheabronchi, which would be consistent with the high incidence of respiratory tract tumors due to dibenzo[*c*,*g*]carbazole.

INTRODUCTION

The important contribution of various polycyclic aromatic hydrocarbons (PAHs) to the carcinogenic potency of complex mixtures is well recognized [7,8,11]. Previous work on the metabolism of carcinogenic PAHs has centered primarily on benzo[*a*]pyrene [6,24,31].

It has been demonstrated that the neutral and basic fractions of these mixtures are carcinogenic when tested in experimental animals [8,28]. These fractions have been found to contain *N*-heterocyclic aromatics [5,22]. Certain of these heterocyclics show considerable carcinogenic potency for the lungs of experimental animals [1,2]. 7*H*-Dibenzo[*c*,*g*]carbazole (7*H*-DB[*c*,*g*]C) (Fig. 1) has produced tumors in the respiratory tract of hamsters [25,26]; 7*H*-DB[*c*,*g*]C given intratracheally (IT) in conjunction

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Abbreviations: 7*H*-DB[*c*,*g*]C, 7*H*-dibenzo[*c*,*g*]carbazole; HPLC, high performance liquid chromatography; IT, intratracheally; IPL, isolated perfused lung preparation; MAC, macrophage pellet; 7-OH-DB[*c*,*g*]C, 7-hydroxydibenzo[*c*,*g*]carbazole, PAHs, polycyclic aromatic hydrocarbons; TB, tracheabronchi; TLC, thin layer chromatography; WO, lavage wash-out fluid remaining following centrifugation to obtain macrophage pellet.

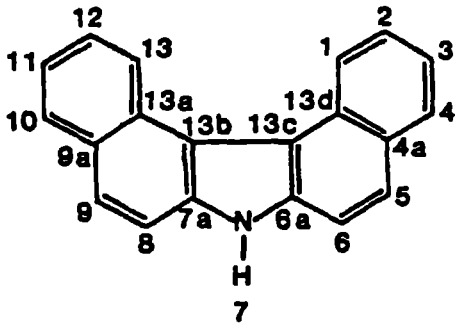


Fig. 1. Structure of 7*H*-dibenzo[*c*,*g*]carbazole.

with haematite dust appears to be a stronger respiratory tract carcinogen than is benzo[*a*]pyrene with comparable doses [11,17].

No work has been done on the metabolism of the carcinogenic *N*-heterocyclic aromatics except for tricycloquinazoline [3,4,12]; therefore, this research investigates the metabolism of the carcinogenic 7*H*-DB[*c*,*g*]C using an isolated perfused lung preparation (IPL) [18].

MATERIALS AND METHODS

Male New Zealand rabbits weighing 4—5 kg were fed single batch standard laboratory chow diets and water ad libitum and were equilibrated for at least 3 weeks in a pesticide-free environment. No anesthesia was given [19] and rabbits were heparinized (1000 I.U./kg) 5 min before cardiac puncture. Immediately following cardiac puncture, the rabbits were killed by CO_2 inhalation and the lungs removed. The equipment, start-up of the perfusion system and preparation of the lungs are described elsewhere [18,19,30].

Two microCuries of [1,4,5,9,10,13,13b,13c-^{14}C]7*H*-DB[*c*,*g*]C (2.7 mCi/mmol), (Amersham, Arlington Heights, IL) diluted with cold 7*H*-DB[*c*,*g*]C (Analabs, North Haven, CT), and synthesized in the laboratory [21] to give 300 μg of 7*H*-DB[*c*,*g*]C (1.78 μCi/μmol), was evaporated gently under nitrogen. The 7*H*-DB[*c*,*g*]C was taken up in 1 ml of ethanolic saline (1 : 1), injected IT on the IPL and deposited at the bifurcation of the lobes. The syringe was rinsed once with an additional 1 ml of saline which was then injected. The purity of 7*H*-DB[*c*,*g*]C was monitored by high performance liquid chromatography methods (HPLC).

Blood samples (5.5 ml) were taken from the perfusion system a minimum of 6 times during the perfusion up to 3 h. The lungs were weighed after removal from the perfusion system and lavaged 3 times with physiological saline (5 ml/g lung tissue); the lung tissue was separated from the trachea-bronchi (TB) and from all extraneous tissue; both the lung tissue and TB were weighed. The extraction procedure and preparation of the organic

extracts of the blood, lung, TB, macrophage pellet (MAC) and lavage wash-out fluid (WO; fluid remaining after centrifuging to obtain macrophage pellet) for HPLC analyses are described elsewhere [18,29,30].

Ten microliter samples were chromatographed on a Varian 8500 HPLC and monitored at 268 nm using a Whatman ODS column, 25 cm × 4.6 mm, 10 μm particle size, with a methanol water gradient, 76—100% methanol, with a flow of 1 ml/min at room temperature. A HPLC chromatogram was recorded using a rat liver microsomal mixture [10,13] of the metabolites (see Fig. 2) once each morning. Fractions from the samples were collected, so that each of the peaks and spaces between peaks were collected individually. The counting efficiency was determined by either using an internal standard or a standard quench curve. The total rate of appearance of metabolites, (ng/g lung) in the blood, was based on a linear regression of a time course study from 0 through at least 90 min. All samples were processed under nitrogen and subdued yellow lighting to minimize photo-oxidation.

Liver microsomes were prepared [10,13] from Sprague—Dawley rats (250 g) injected IP with 3-methylcholanthrene (20 mg/kg) and the pellet was frozen at −80°C at a concentration of 10 mg protein/ml [14] in 0.1 M Tris buffer (pH 7.4). The assay [10] was carried out in a total of 4 ml 0.1 M Tris buffer (pH 7.4) at 37°C for 1 h using a regenerating NADP/glucose 6-phosphate system with total protein varying from 1.6 mg to

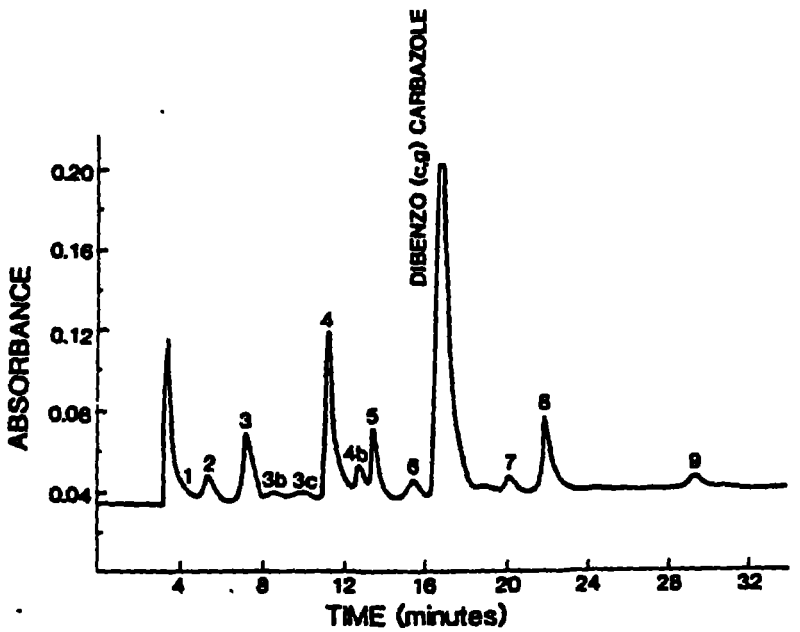


Fig. 2. HPLC chromatogram of 7*H*-dibenzo[c,*g*]carbazole and its metabolites.

6.4 mg; ethyl acetate extracts were dried over $MgSO_4$, evaporated under N_2 and frozen until HPLC analyses (Fig. 2). Acetylated derivatives of amine and/or hydroxyl groups for mass spectral analyses were prepared by dissolving metabolite(s) in ethyl acetate, adding 15 μl of pyridine and 30 μl of acetic anhydride, heating at 45°C for 30 min [20] and evaporating to dryness. All solvents were re-distilled except methanol (Fisher Scientific, NJ, spectroanalyzed). All ^{14}C scintillation counting was performed on Tri-Carb Packard Liquid Scintillation Spectrometers and corrected fluorescence, ultraviolet, infrared and mass spectra were recorded on an American Instrument Company spectrophotofluorometer, Beckman DB-GT, and Perkin Elmer 457 infrared spectrophotometers and Finnegans 3200 and 3300 mass spectrometers, respectively.

RESULTS AND DISCUSSION

The results indicate that the rate of appearance of metabolites in the blood for 7*H*-DB[*c*,*g*]C (300 μg, 1.12 μmol, given IT, Table 1(A)) is twice as rapid as benzo[*a*]pyrene (316 μg, 1.25 μmol, given IT; TLC data, 256 ±

TABLE 1

METABOLISM AND DISTRIBUTION OF 7*H*-DIBENZO[*c*,*g*]CARBAZOLE AND ITS METABOLITES IN THE ISOLATED PERFUSED LUNG ± S.E.

A		B		
Total rate of appearance of metabolites in the blood[a] (ng/g lung/h)		Distribution of parent compound and metabolites in tissue at the end of the perfusion[b]		
765 ± 176		Total percent of 7*H*-DB[*c*,*g*]C remaining		
		32.0 ± 8.2		
Percent of total rate of metabolism		Percent of total compound in each tissue		
		7*H*-DB[*c*,*g*]C		Total metabolite
Metabolite				
1	5.6 ± 1.9	Blood	7.9 ± 1.4	21.7 ± 5.0
2	6.8 ± 1.4	TB	4.3 ± 1.0	8.1 ± 0.9
3	10.9 ± 2.2	MAC	1.4 ± 0.5	1.4 ± 0.3
3b,3c	3.3 ± 0.8	WO	6.0 ± 2.7	8.1 ± 0.6
4	2.3 ± 0.4	Lung	12.3 ± 4.1	28.8 ± 4.3
5	1.0 ± 0.3			
6	1.1 ± 0.4			
7	3.1 ± 1.5			
8	2.6 ± 1.1			
9	1.4 ± 0.5			
Non-extractable	56.6 ± 4.4			

[a]Five experiments; 2 μCi; 300 μg/exp.
[b]Three exp. run at least 120 min.

37 ng/g lung/h, ± S.E. for 9 experiments; HPLC data, 334 ± 40 ng/g lung/h, ± S.E. for 4 experiments) in the IPL. This may be partially a function of the increased solubility of 7*H*-DB[*c,g*]C compared with other PAHs [11] in aqueous media in addition to increased enzymatic activation.

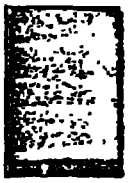
HPLC methods have been used in isolating 9 metabolites in the rat liver microsome mixture (Fig. 2) which are used to identify the metabolites formed in the isolated perfused lung (Table1(A)). It should be noted that qualitatively similar metabolites of benzo[*a*]pyrene are found in various species, as well as in various organs such as liver and lung, but there are quantitative differences [10,23]. The same is true for 7*H*-DB[*c,g*]C; the metabolite pattern (Table 1(A) using radiolabeled compound indicates that metabolites 1—3 are produced most readily in the IPL while metabolites 3 and 4 are produced most readily in rat liver microsomes (not shown).

Infrared spectrum of 7*H*-DB[*c,g*]C (m.p. 156°C) in a halo-carbon mull produces a sharp band at 3420 cm^{-1} indicative of an N—*H* stretching vibration. The infrared spectrum of metabolite 3 (decomposes > 240°C) in chloroform produces a broad strong band at 3450 cm^{-1} indicative of a hydrogen bonded O—H stretching vibration. Fluorescence emission spectra in methanol for 7*H*-DB[*c,g*]C and metabolite 3 have peaks at 369 and 387 nm and at 392 and 405 nm, respectively; this shift to longer wavelengths for metabolite 3 is consistent with the increase in polarity due to a hydroxyl functional group [15, D. Warshawsky, unpublished data].

Mass spectral analysis of 7*H*-DB[*c,g*]C shows a molecular ion at *m/e* 267 while the acetylated derivative of 7*H*-DB[*c,g*]C produces a molecular ion at *m/e* 309 (plus 42 mol. wt, relative abundance of 3.2) followed by a fragmentation ion at *m/e* 267 (relative abundance of 100). This indicates that the nitrogen is acetylated. Metabolite 3 produces a molecular ion at *m/e* 283 that is indicative of an oxygenated derivative (plus 16 mol. wt). Acetylation of metabolite 3 produces a molecular ion at *m/e* 325 (relative abundance of 60) followed by a fragmentation ion at *m/e* 283 (relative abundance of 100). Since only 1 position on 7*H*-DB[*c,g*]C has been derivatized, a hydroxylated nitrogen must be the functional group that has been acetylated. Therefore, metabolite 3 is 7-hydroxydibenzo[*c,g*]carbazole (7-OH-DB[*c,g*]C). The free valence values [27] (ability of each atom to undergo electrophilic attack) are consistent with the readily formed 7-OH-DB[*c,g*]C: atom(s) of 7*H*-DB[*c,g*]C (Fig. 1), free valence; 7, 1.020; 5,9, 0.461; 4,10, 0.453; 1,3, 0.444; 6,8, 0.424; 2,12, 0.403.

IPL distribution data (Table 1(B)) indicate that 32.0% ± 8.2 of 7*H*-DB-[*c,g*]C remains at the end of the IPL experiment compared to 65% ± 4.3 for benzo[*a*]pyrene (not shown). There is a substantial percentage of 7*H*-DB[*c,g*]C and metabolites, in particular 7-OH-DB[*c,g*]C, remaining in TB as well as in lung and perfusate.

The IPL results indicate that 7*H*-DB[*c,g*]C is metabolized readily following IT administration in the lung, but that a substantial amount of



7*H*-DB[*c,g*]C and total metabolite are found in the TB. This suggests that the pulmonary aveolar macrophages may deposit compounds in the TB [9] which would be consistent with the high incidence of respiratory tract tumors in the trachea and bronchi due to 7*H*-DB[*c,g*]C [25,26]. 7-OH-DB-[*c,g*]C, which is found in the TB in the largest quantities, may be the initial step in the formation of the ultimate metabolite of 7*H*-DB[*c,g*]C [12,16].

We are presently characterizing all of the metabolites and determining the mutagenic activity of each using the *Salmonella typhimurium* microsomal assay.

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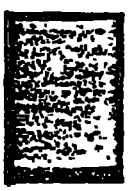

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